Manipulation of the Blue Light Photoreceptor Cryptochrome 2 in Tomato Affects Vegetative Development, Flowering Time, and Fruit Antioxidant Content

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Cryptochromes are blue light photoreceptors found in plants, bacteria, and animals. In Arabidopsis, cryptochrome 2 (cry2) is involved primarily in the control of flowering time and in photomorphogenesis under low-fluence light. No data on the function of cry2 are available in plants, apart from Arabidopsis (Arabidopsis thaliana). Expression of the tomato (Solanum lycopersicum) CRY2 gene was altered through a combination of transgenic overexpression and virus-induced gene silencing. Tomato CRY2 overexpressors show phenotypes similar to but distinct from their Arabidopsis counterparts (hypocotyl and internode shortening under both low- and high-fluence blue light), but also several novel ones, including a high-pigment phenotype, resulting in overproduction of anthocyanins and chlorophyll in leaves and of flavonoids and lycopene in fruits. The accumulation of lycopene in fruits is accompanied by the decreased expression of lycopene-β-cyclase genes. CRY2 overexpression causes an unexpected delay in flowering, observed under both short- and long-day conditions, and an increased outgrowth of axillary branches. Virus-induced gene silencing of CRY2 results in a reversion of leaf anthocyanin accumulation, of internode shortening, and of late flowering in CRY2-overexpressing plants, whereas in wild-type plants it causes a minor internode elongation.

Cryptochromes are flavin-containing blue light photoreceptors, first discovered in plants. The first cryptochrome gene was isolated through the insertional cloning of an Arabidopsis (Arabidopsis thaliana) mutant allelic to hy4 (Ahmad and Cashmore, 1993). Our knowledge of the function of higher plant cryptochromes relies almost exclusively on the study of a single plant, Arabidopsis. Arabidopsis contains at least three cryptochromes, two (cryptochrome 1 [cry1] and cry2) localized predominantly in the nucleus and the cytoplasm (Lin and Shalitin, 2003) and one (cry3) in the organelles (Kleine et al., 2003). Functional characterization of Arabidopsis mutants has shown that cry1 is mainly involved in the control of photomorphogenesis, including hypocotyl elongation and anthocyanin biosynthesis, while cry2 is mainly involved in the control of flowering time and of hypocotyl elongation (Guo et al., 1998; Lin et al., 1998; Lin and Shalitin, 2003). The role of cry2 in Arabidopsis is strictly dependent on light fluence and photoperiod; cry2− mutants and CRY2 overexpressors have, respectively, long and short hypocotyls under low, but not high, blue light intensities. cry2− mutants flower later than the wild type in long-day but not short-day conditions, and CRY2 overexpressors flower earlier than the wild type in short-day but not long-day conditions (Guo et al., 1998; Lin and Shalitin, 2003). A naturally found, gain-of-function allele of CRY2 is responsible for the early flowering in short days of the Cape Verde ecotype of Arabidopsis, with respect to its northern European counterparts (El-Din El-Assal et al., 2001, 2003).

The developmental patterns of Arabidopsis and tomato (Solanum lycopersicum) are different. Arabidopsis shows a monopodial type of growth (Schmitz and Theres, 1999) in which the shoot apical meristem (SAM) gives rise to vegetative metamers, each composed of a very short internode, a rosette leaf, and a bud. Upon transition to flowering, the SAM is transformed into an inflorescence meristem (IM), giving rise to an inflorescence with cauline leaves, elongated internodes, and flowers. Axillary meristems are rec-
ognizable in Arabidopsis only after the transition to flowering. Tomato, or at least its indeterminate varieties, such as the Moneymaker cultivar used in this study, shows a sympodial type of development. Early on, the SAM gives rise to vegetative metamers similar to Arabidopsis, the major differences being a much more elongated internode and a clearly recognizable axillary meristem, located at the axil of each leaf. After 7 to 11 metamers, the SAM is transformed into an IM, and the axillary meristem located immediately below it becomes the novel SAM. This novel shoot apex forms approximately three metamers and is then transformed into an IM, while a new axillary meristem becomes the SAM (and so on). The transition from a SAM to an IM is tightly regulated by daylength in Arabidopsis, with most Arabidopsis ecotypes showing a facultative long-day requirement for flowering, whereas no major effects of photoperiod on tomato flowering time have been reported (Koornneef et al., 1998).

Another fundamental difference between the two plants is the development of a fleshy fruit in tomato, whose function is to attract animals involved in seed dispersion. After a period of cell division and cell expansion, leading to the mature green stage, the fruit undergoes a series of changes, controlled by ethylene and collectively known as ripening, which involve the transformation of chloroplasts into chromoplasts (containing high levels of the linear carotene lycopene), the softening of the fruit, and the accumulation of a series of compounds increasing fruit palatability. At the gene expression level, this change involves the differential regulation of genes involved in the biosynthesis of ethylene, carotenoids, and the degradation of cell wall components (Giovannoni, 2001).

We have started the molecular and functional characterization of the tomato cryptochrome gene family. To date, two CRY1, one CRY2, and one CRY3 gene have been isolated in tomato (Perrotta et al., 2000, 2001; G. Perrotta, unpublished data). The role of one of the CRY1 genes, CRY1a, has been elucidated through the use of antisense (Ninu et al., 1999) and mutant (Weller et al., 2001) plants. cry1a controls seedling photomorphogenesis, anthocyanin accumulation, and adult plant development. No effects of cry1a on flowering time or fruit pigmentation have been observed. Little is known about the functional role of the remaining tomato cryptochromes, mainly due to the lack of mutants. In this article, we present the phenotypic characterization of tomato plants in which CRY2 expression has been modified through a combination of transgenic overexpression and virus-induced gene silencing (VIGS).

RESULTS

Effects of CRY2 Overexpression on Vegetative Growth

To alter the expression of CRY2, we transformed tomato (cv Moneymaker) with a 35S::CRY2 construct carrying the tomato CRY2 cDNA (Perrotta et al., 2000) under the control of the cauliflower mosaic virus 35S promoter. Several independent transformants were regenerated, selfed, and the T1 seeds collected. Since one of the phenotypes of Arabidopsis CRY2 overexpressors is hypocotyl shortening under low-irradiance blue light (Lin et al., 1998), seeds were germinated under blue and red light (2 μmol m⁻² s⁻¹). Seven out of 10 transgenic lines showed a short-hypocotyl phenotype under blue but not under red light, segregating in an approximately 3:1 ratio. The hypocotyl lengths for

Figure 1. cry2 overexpression produces a short-hypocotyl phenotype under blue light. A and B, Hypocotyl lengths of the wild type and of three independent transgenic lines segregating for the 35S::CRY2 transgene. Seeds were germinated for 7 d under continuous low-irradiance (2 μmol m⁻² s⁻¹) blue (A) or red (B) light. Each asterisk represents an individual seedling. C, Relative expression, normalized for the actin transcript, of CRY transcripts in “short” (2-cm) seedlings from A.
three representative lines are shown in Figure 1, A and B. In all three lines, the short-hypocotyl phenotype cosegregated with the presence of the 35S::Cry2 transgene (data not shown), confirming that the phenotype was indeed due to cry2 overexpression and not to somaclonal effects arising during the transformation procedure. We measured transcript levels for CRY1a, CRY1b, and CRY2 via real-time quantitative reverse transcription (RT)-PCR (RT-QPCR) in transgenic seedlings (Fig. 1B); CRY2 was overexpressed 5- to 6-fold in the seedlings showing the short-hypocotyl phenotype, whereas the other two cryptochromes showed essentially normal transcript levels.

We then grew CRY2-overexpressing (CRY2-OX) plants under high-irradiance white light (40 μmol m⁻² s⁻¹) in a long-day photoperiod (16 h light/8 h dark). These plants showed several additional phenotypes. The seedlings accumulated high levels of anthocyanins in all organs, including roots (Fig. 2D). Adult plants were dwarfed (Fig. 2A), had darker green leaves with anthocyanin-accumulating veins (Fig. 2B), and showed an abnormal outgrowth of axillary meristems (Fig. 2C). Spectrophotometric measurements indicated a generalized pigment increase in leaves of the transgenic lines, very evident (up to 3-fold) for anthocyanins and less so for chlorophylls and carotenoids (Fig. 3, A–C). The internodes of adult plants were significantly shorter (Figs. 2C and 3D).

Homozygous transgenic seed populations were obtained from lines 52.3 and 52.8 and germinated un-
der different light qualities and intensities. CRY2-OX seedlings showed, with respect to their wild-type counterparts, reduced hypocotyl length and elevated anthocyanin content when grown under white and blue but not red light (Fig. 4, A and B). Increasing the fluence rate (from 2–16 μmol m⁻² s⁻¹ for blue and from 5–40 μmol m⁻² s⁻¹ for white) magnified both responses in CRY2-OX seedlings. The strongest responses, both for hypocotyl shortening and for anthocyanin accumulation, were observed under high-irradiance white light, suggesting that blue light alone is insufficient for complete responses. Under these conditions, all organs of CRY2-OX seedlings, including roots, showed strong anthocyanin pigmentation (Fig. 2D).

Collectively, these results indicate that CRY2 overexpression in tomato produces a series of alterations in

Figure 3. Pigmentation and internode length (average of three transgenic plants grown in the same conditions as in Fig. 2). A, Leaf anthocyanins. B, Leaf chlorophylls. C, Leaf carotenoids. D, Average length of the first five internodes. E, Lycopene content in ripe fruit pericarps from greenhouse-grown plants.

Figure 4. Seedling phenotypes. (Homozygous, 7-d-old transgenic seedlings of lines 52.3 and 52.8 were induced to germination with a 12-h pulse of high-irradiance white light and then grown for 7 d under different continuous light conditions.) A, Hypocotyl length. B, Anthocyanin content. W, White; B, blue; R, red; D, dark; H, high irradiance (40 μmol m⁻² s⁻¹ for W and 15 μmol m⁻² s⁻¹ for B and R); L, low irradiance (5 μmol m⁻² s⁻¹ for W and 2 μmol m⁻² s⁻¹ for B and R). For further details, see "Materials and Methods."
vegetative growth, some of which (hypocotyl shortening under high irradiances, anthocyanin accumulation, and dwarfism of adult plants) are more similar to those of Arabidopsis CRY1, rather than CRY2, overexpressors (Lin et al., 1996, 1998). Yet another phenotype (induction of axillary branch growth) has not been described before in Arabidopsis CRY1 or CRY2 overexpressors, whereas tomato cry1 mutants show decreased growth of axillary branches (Weller et al., 2001). These observations suggest that, in tomato, both cry1 and cry2 play stimulatory roles in the growth of axillary branches.

Effects on Flowering Time

In Arabidopsis, cry2 plays a major role in the control of flowering time; cry2– mutants flower later than the wild type under long but not short days, and CRY2 overexpressors flower earlier than the wild-type under short but not long days (Guo et al., 1998). Although tomato has a different developmental pattern than Arabidopsis, it is possible to measure flowering time as the time elapsed between seed sowing and anthesis of the first flower.

Surprisingly, all three CRY2-OX lines show retardation, instead of anticipation, of flowering under both short and long days (Table I). This retardation in time, however, does not correspond to a change in the number of vegetative metamers that are formed before the first inflorescence appears (Table I).

Effects on Fruit Antioxidants

Tomato fruit pigmentation is influenced by light perceived from the environment. Fruit-localized phytochromes positively influence the accumulation of lycopene (Alba et al., 2000). Tomato orthologs of genes that in Arabidopsis are involved in light signal transduction, such as LeHy5, Hp2/LeDet1, Hp1, and LeCop1-like, have positive or negative effects on tomato fruit pigmentation, in agreement with their proposed positive or negative signaling roles in Arabidopsis photomorphogenesis (Mustilli et al., 1999; Liu et al., 2004). Regarding the role of specific photoreceptors, phytochrome B2 positively regulates the pigmentation of mature green fruits (Weller et al., 2001), whereas cry1a manipulation through antisense silencing, mutation, or overexpression has only minor effects on fruit pigmentation (Ninu et al., 1999; Weller et al., 2001).

Upon visual inspection, fruits of the three CRY2-OX lines show increased pigmentation, both at the immature green and at the red ripe stage (Fig. 2, E and F). We spectrophotometrically measured the levels of lycopene, the major red pigment and lipophilic antioxidant in tomato fruits, at the red ripe stage. The data show an approximately 1.5- to 2-fold increase in this pigment (Fig. 3E).

The pigment (carotenoid and flavonoid) content of the pericarp of red ripe fruits of line 52.3 was also analyzed by HPLC (Table II). Again, lycopene shows a significant (1.7-fold) increase, with other carotenoids (if-carotene and lutein) showing more modest and variable (for β-carotene) increases. Water-soluble antioxidants (phenylpropanoids, flavonoids) also show a significant increase (2.9-fold), with rutin, the major flavonoid, showing the highest increase (4.3-fold).

It has been shown previously that accumulation of lycopene in ripening tomato fruits is associated with the increase in transcript levels of lycopene biosynthetic genes, like phytoene synthase 1 (Psy1) and phytoene desaturase (Pds; Giuliano et al., 1993), and the repression of lycopene-catabolizing genes like lycopene β-cyclase (β-Lcy; Pecker et al., 1996). The mRNA levels of these genes, as well as of other genes for carotenoid biosynthesis and of cryptochrome genes, were measured at the red ripe stage through RT-QPCR. Transcripts involved in lycopene synthesis (Psy1, Zds), as well as CRY1a and CRY1b transcripts, do not show significant variations in CRY2-OX fruits with respect to their wild-type counterparts (Table III). Actually, the Pds transcript shows a slight repression instead of an induction, consistent with the model that we had previously proposed, i.e. that the activity of the Pds promoter is feedback regulated by carotenoid levels (Corona et al., 1996). Instead, transcripts of the two genes involved in lycopene cyclization (β-Lcy, B) show

### Table I. Flowering time of wild-type and CRY2-OX plants grown under high-irradiance (40 μmol m⁻² s⁻¹) white light

<table>
<thead>
<tr>
<th>Line</th>
<th>Days to Flowering</th>
<th>Leaves before First Flower</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Long Days)</td>
<td>(Short Days)</td>
</tr>
<tr>
<td>Wild type</td>
<td>55 ± 6</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>52.3</td>
<td>71 ± 3</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>52.5</td>
<td>64 ± 9</td>
<td>n.d.</td>
</tr>
<tr>
<td>52.8</td>
<td>68 ± 5</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*Not determined.*

### Table II. Carotenoid/flavonoid content in ripe fruit pericarps from greenhouse-grown plants

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Wild Type</th>
<th>CRY2-OX 52.3</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lycopene</td>
<td>775 ± 110</td>
<td>1,353 ± 84</td>
<td>1.7</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>78 ± 8</td>
<td>101 ± 31</td>
<td>1.3</td>
</tr>
<tr>
<td>Lutein</td>
<td>23 ± 1</td>
<td>36 ± 4.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Total</td>
<td>876 ± 113</td>
<td>1,490 ± 114</td>
<td>1.7</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>0.14 ± 0.05</td>
<td>0.42 ± 0.10</td>
<td>3.1</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.06 ± 0.02</td>
<td>0.21 ± 0.05</td>
<td>3.5</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.80 ± 0.29</td>
<td>3.48 ± 0.95</td>
<td>4.3</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.38 ± 0.10</td>
<td>0.38 ± 0.17</td>
<td>1.0</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.48 ± 0.05</td>
<td>0.83 ± 0.21</td>
<td>1.7</td>
</tr>
<tr>
<td>chalcone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.85 ± 0.38</td>
<td>5.31 ± 1.14</td>
<td>2.9</td>
</tr>
</tbody>
</table>
an approximately 2-fold repression in CRY2-OX fruits. We and others have shown previously that fruit-specific silencing of either of the two transcripts (β-Lcy or B) results in increased lycopene levels (Ronen et al., 2000; Rosati et al., 2000). Thus, the data are consistent with the idea that CRY2 overexpression increases fruit lycopene content through the repression of lycopene cyclase genes rather than through the induction of earlier genes in the carotenoid pathway.

**Virus-Induced Gene Silencing**

VIGS has been advocated as a tool for large-scale functional genomics in plants (Baulcombe, 1999). It has been used for the functional characterization of several plant genes, including those involved in responses to pathogens or in primary or secondary metabolism, but not genes for plant photosensory receptors. Potato virus X (PVX) has been used extensively for inducing VIGS in *Nicotiana benthamiana* (Angell and Baulcombe, 1999). We constructed a PVX-based silencing vector (PVX::CRY2), containing the 3' nontranslated region of the CRY2 transcript cloned in the pPVX201 plasmid (Baulcombe et al., 1995). This region was chosen because it shows negligible homology to the CRY1 transcripts, thus minimizing the possibility of gene silencing. As no reports are available on the use of PVX for inducing VIGS in tomato, a DNA-based infection method was developed (see “Materials and Methods”). Wild-type and CRY2-OX plants grown under long-day conditions were infected with PVX::CRY2 and, as a control, with PVX alone. At 3 weeks postinfection (5 weeks post-sowing), the silencing of the CRY2 transcript in PVX::CRY2-infected plants was approximately 3-fold in wild-type and 5-fold in CRY2-OX plants (Table IV). The higher levels of silencing observed in transgenic plants are in agreement with what was observed previously (Baulcombe, 1999). The levels of the CRY1a and CRY1b transcripts were unaltered in PVX::CRY2-infected plants, as were those of all cryptochrome genes after infection with PVX alone (Table IV).

The silencing of the CRY2 transgene in CRY2-OX plants was accompanied by a reversion of several of the CRY2-OX phenotypes: three phenotypes that were clearly suppressed by silencing but not by infection with PVX alone were internode shortening (Figs. 2G and 5A), anthocyanin pigmentation of leaf veins (Fig. 2H), and late flowering (Fig. 5B). Infection with PVX alone caused a significant degree of internode shortening both in wild-type and CRY2-OX plants (Fig. 5A). Therefore, the minor internode elongation observed in CRY2-silenced, wild-type plants (Fig. 5A) is probably the result of two contrasting effects: internode shortening induced by PVX infection and internode elongation induced by silencing of CRY2. No clear effects of CRY2 silencing on flowering time of wild-type plants were observed.

The lateral branching phenotype was also suppressed in silenced plants. However, the interpretation of the data was complicated by the fact that infection with PVX alone has a clear inhibitory effect on the growth of axillary branches (data not shown).

**DISCUSSION**

In Arabidopsis, cry2 plays multiple roles in regulating photomorphogenesis at low irradiances and flowering time (Lin and Shalitin, 2003; Yanovsky and Kay, 2003). CRY2 overexpression in tomato results in several photomorphogenic and flowering phenotypes, some of which are expected on the basis of the experience gained with Arabidopsis while others are novel (Fig. 6). Similar to their Arabidopsis counterparts, tomato CRY2 overexpressors show a light quality-dependent short-hypocotyl phenotype under low irradiances (Lin et al., 1998). However, the enhancement of the short-hypocotyl phenotype at high irradiances, the anthocyanin accumulation, and the dwarfism of adult plants observed in CRY2-OX tomato are associated with CRY1, rather than CRY2, overexpression in Arabidopsis (Lin et al., 1996, 1998). We have previously shown that impairment of tomato cry1a function reduces anthocyanin levels and increases internode and hypocotyl length (Ninu et al., 1999; Weller et al., 2001). The presence of cry1-like phenotypes in cry2-overexpressing lines suggests that cry2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Line</th>
<th>Uninfected</th>
<th>PVX Infected</th>
<th>Silenced</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRY2</td>
<td>Wild type</td>
<td>1.0 ± 0.3</td>
<td>1.12 ± 0.07</td>
<td>0.3 ± 0.11</td>
</tr>
<tr>
<td>CRY2-OX</td>
<td>5.4 ± 1.7</td>
<td>4.6 ± 1.0</td>
<td>0.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>CRY1a</td>
<td>Wild type</td>
<td>1.0 ± 0.02</td>
<td>1.15 ± 0.2</td>
<td>0.99 ± 0.2</td>
</tr>
<tr>
<td>CRY2-OX</td>
<td>0.89 ± 0.17</td>
<td>1.11 ± 0.2</td>
<td>0.83 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>CRY1b</td>
<td>Wild type</td>
<td>1.0 ± 0.15</td>
<td>1.21 ± 0.24</td>
<td>0.83 ± 0.14</td>
</tr>
<tr>
<td>CRY2-OX</td>
<td>1.16 ± 0.2</td>
<td>1.19 ± 0.28</td>
<td>0.8 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>
plays roles partially redundant with those of cry1 in the control of tomato photomorphogenesis.

The pigment content of CRY2-OX leaves and fruits suggests that cry2 controls some aspect of pigment biosynthesis/accumulation. It is possible that this control is at least partially exerted at the transcriptional level. We had previously shown that the Arabidopsis nuclear Psy gene, encoding the first dedicated step in the plastid-localized carotenoid biosynthesis pathway, is positively regulated by blue light through an unidentified photoreceptor (von Lintig et al., 1997). CRY2 overexpression in tomato increases flavonoid and lycopene levels in ripe fruits. This phenotype is reminiscent of the tomato high pigment mutants hp1 and hp2, which display shortened hypocotyls and internodes, anthocyanin accumulation, and highly pigmented fruits. Hp1 and Hp2 encode the tomato orthologs of negative regulators of light signal transduction in Arabidopsis: Det1 and Ddb1 (Mustilli et al., 1999; Liu et al., 2004). Our data suggest that, consistent with the observed accumulation of lycopene, Cry2 overexpression represses two genes in the carotenoid biosynthetic pathway (lycopene β-cyclases) in fruits. Previous findings had indicated that transgenic silencing of these genes results in increased fruit lycopene levels (Ronen et al., 2000; Rosati et al., 2000). Microarray profiling of CRY2-OX plants is under way to determine which genes, in pigment biosynthesis as well as in different biochemical pathways, are regulated by CRY2 overexpression.

Several laboratories, including ours, have achieved the targeted increase in tomato fruits of individual antioxidant compounds, such as the carotenoids lycopene, β-carotene, and zeaxanthin and the flavonoids rutin and chlorogenic acid, through metabolic engineering of individual biosynthetic steps (Romer et al., 2000; Rosati et al., 2000; Muir et al., 2001; Dharmapuri et al., 2002; Fraser et al., 2002; Niggeweg et al., 2004) or of regulatory transcription factors (Bovy et al., 2002). Manipulation of light-sensing pathways (Liu et al., 2004; this study) is, to our knowledge, a novel strategy to achieve the simultaneous increase of multiple antioxidant compounds impacting on fruit nutritional value. However, as a word of caution, we wish to add that metabolomic profiling of CRY2-OX fruits shows a high degree of metabolic perturbation (L. Giliberto, A. Fernie, N. Schauer, and G. Giuliano, unpublished data) whose nutritional impact has still to be carefully evaluated.

Flowering time is an important trait, influencing both the time it takes for a plant to reproduce in the wild and the time it takes to produce harvestable fruits and seeds for human consumption. In Arabidopsis,
a long-day plant, the photoreceptors cry2 and phytochrome A act positively and phytochrome B acts negatively to regulate flowering in response to light (Koornneef et al., 1998; Mockler et al., 2003; Valverde et al., 2004). Signals from these photoreceptors and those from the endogenous circadian clock are integrated at the level of CONSTANS (CO), a transcriptional regulator positively influencing the mRNA levels of FLOWERING LOCUS T (FT), which in turn, when reaching certain thresholds, activate the transition from vegetative growth to flowering (Yanovsky and Kay, 2002, 2003; Valverde et al., 2004). In rice (Oryza sativa), a short-day plant, phytochromes but not cry2 photoreceptors have been implicated in the photoperiodic control of flowering (Izawa et al., 2002; Hayama et al., 2003). The short-day phenotype of rice has been suggested to derive from a different regulatory relationship between CO and FT, which is inductive in Arabidopsis and inhibitory in rice (Hayama et al., 2003). Cultivated tomato behaves differently from both Arabidopsis and rice: It is generally considered a day neutral plant, although it flowers earlier when daylength is increased without proportionally decreasing light intensity (Table I). An interesting question is how, in a day-neutral plant, cry2 overexpression can delay flowering in a photoperiod-independent fashion, without influencing the number of leaves made before the first inflorescence appears. Further experiments measuring the activity of CO, FT, and meristem identity genes in plants with altered cry2 activity are needed to clarify this point.

Another important agricultural trait is axillary branching. In tomato, the excessive outgrowth of axillary branches negatively affects fruit production so that they have to be manually removed, increasing labor costs. Mutants in some tomato genes, such as lateral suppressor, prevent the initiation of axillary meristems (Schumacher et al., 1999). At variance with the role of such genes, cry2 overexpression releases the inhibition of axillary meristems—a phenomenon known as apical dominance—rather than altering their number or position. Apical dominance is a complex phenomenon involving the hormones auxin and cytokinin (Chatfield et al., 2000) as well as other, yet unidentified signaling molecules (Booker et al., 2004). We have not found gross alterations of overall hormone levels in cry2-OX plants. However, cytokinin has been shown to act over short distances in paracrine signaling (Faiss et al., 1997). Thus, it is possible that more localized alterations in hormone levels have escaped our attention. An alternative possibility is that cry2 regulates a signal at or downstream of the hormone-sensing step. Both possibilities are presently being investigated.

This is the first time, to our knowledge, that VIGS has been used to functionally study a plant photosensory receptor. In some cases (flowering time, internode length, anthocyanin accumulation), VIGS clearly reverted the cry2-OX phenotypes, whereas PVX infection did not. In other cases (chlorophyll and carotenoid accumulation, lateral branching), PVX infection itself interfered with the phenotypes studied, complicating the interpretation of the results. Two other drawbacks of the PVX system are its inherent instability in tomato, the low levels of silencing obtained in wild-type plants, and the variable silencing observed in fruits. Other silencing vectors have now been developed, which may overcome some of these problems (Liu et al., 2002).

In conclusion, cry2 is a central player in tomato development. Its manipulation through transgenic overexpression and VIGS results in the alteration of a large set of developmental and biochemical responses both in vegetative and reproductive tissues (Fig. 6). In Arabidopsis, cry2 controls photomorphic responses through the interaction with the photomorphogenic repressor Cop1, resulting in a modulation of Hy5 abundance (Wang et al., 2001) and flowering time through the modulation of CO mRNA and protein levels (Yanovsky and Kay, 2002; Valverde et al., 2004). Efforts are under way to determine the signal transduction pathway in tomato.

MATERIALS AND METHODS

Standard molecular biology protocols were followed as described (Sambrook et al., 1989). The cry2 full-length cDNA of tomato (Solanum lycopersicum; Perrotta et al., 2000) was cloned in the Smal and SacI sites of the Agrobacterium-based vector pBI121 (Jefferson et al., 1987), under the control of the cauliflower mosaic virus 35S promoter. Agrobacterium-mediated transformation was performed according to a published protocol (van Roekel et al., 1993). To establish truly independent transformation events, only one regenerant was taken from every cocultivated tomato explant. For fruit and seed production, plants were grown in the greenhouse under controlled conditions (16 h light, 25°C/8 h dark, 20°C).

All germination and growth experiments were conducted in growth chambers at a constant temperature of 25°C. Light sources were as follows: white was provided by Osram (Munich) 11-860 lamps and Osram Fluora 77 lamps (2:1 ratio). Blue was provided by Osram 67 lamps, additionally filtered through a Lee (Hampshire, UK) Dark Blue plastic filter (ref. 119). Red was provided by Osram 60 lamps, additionally filtered through a Lee Primary Red plastic filter (ref. 106). Irradiances were adjusted by using Lee neutral density filters to 40 and 5 μmol m \(^{-2}\) s \(^{-1}\) (high and low irradiance) for white, and 15 and 2 μmol m \(^{-2}\) s \(^{-1}\) (high and low irradiance) for blue and red.

Seeds were harvested from ripe fruits (10–15 d after breaker stage), treated for 20 min in 100 mM HCl, washed extensively in tap water, dried overnight on filter paper, and stored at room temperature in capped tubes over silica gel. For measuring hypocotyl lengths, seeds were imbibed on two layers of filter paper, moistened with tap water, in Magenta boxes (Sigma, Milan). Germination was induced with 16 h of high-fluence white light, ensuring a homogeneous germination rate. The seeds were transferred under the various light conditions for 7 d.

Data (pigment content, HPLC profiles, internode length, days to anthesis, transcript levels) were collected from at least three independent experimental replicates. For hypocotyl length, at least 12 seedlings per experiment were measured in duplicate experiments. Total RNA was isolated using a published protocol (Lopez-Gomez and Gomez-Lim, 1992); retrotranscribed using oligo(dT)\(_{16}\), and then quantified through real-time quantitative PCR using an Applied Biosystems 7000 and the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The oligonucleotides used (designed using the Primer Express program) were the following: CR2: GGGATGCTTGAATGAGCTTTAAT and CCTACTTGGACTATTTAGC; CRY1a: GGGATGCTTGAATGAGCTTTAAT and CCTACTTGGACTATTTAGC; CRY1b: GGGATGCTTGAATGAGCTTTAAT and CCTACTTGGACTATTTAGC; CRY2: GGGATGCTTGAATGAGCTTTAAT and CCTACTTGGACTATTTAGC; CRY3: GGGATGCTTGAATGAGCTTTAAT and CCTACTTGGACTATTTAGC; CRY4: GGGATGCTTGAATGAGCTTTAAT and CCTACTTGGACTATTTAGC.

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LITERATURE CITED


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